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In Vivo Study of the Efficacy of the Aromatic Water of *Zataria multiflora* on Hydatid Cysts

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Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) were employed to determine the chemical composition of the essential oil (EO) from aromatic water (AW) of *Zataria multiflora*. Thymol (66.9%), carvacrol (15.2%), and carvone (7.3%) were found to be the major EO constituents. Eighty laboratory BALB/c mice were infected intraperitoneally by injection of 1,500 viable protoscolices and were divided into prevention (40 mice) and therapeutic (40 mice) groups. To prove the preventive effect of the *Z. multiflora* AW on development of hydatid cysts, the 40 infected mice were allocated into three treatment groups, including the albendazole group (10 mice that received 150 mg/kg body weight/day for 10 days), the *Z. multiflora* AW group (15 mice that received 20 ml/liter in drinking water for 8 months), and a control group (15 mice that received no treatment). To estimate the therapeutic effect of the *Z. multiflora* AW on the hydatid cyst, after 8 months of infection, the 15 remaining mice were allocated into three experimental treatment groups of five animals each, including the albendazole group (300 mg/kg/day for 20 days), *Z. multiflora* AW group (40 ml/liter in drinking water for 30 days), and control group (no treatment). All mice were then euthanized, and the sizes and weights of the cysts as well as their ultrastructural changes were investigated. The weights and sizes of the hydatid cysts significantly decreased upon treatment with the *Z. multiflora* AW in both the preventive and therapeutic groups ($P < 0.05$). The results of scanning electron microscopy also showed considerable damage in the germinal layer of the hydatid cysts recovered from the treated animals.

Echinococcus granulosus is a taeniid tapeworm that occurs in the small intestine of definitive hosts, notably dogs and occasionally other carnivores. The larval stage (metacestode) of *E. granulosus* causes cystic echinococcosis (CE) (also known as cystic hydatidosis) in humans and livestock and represents an important zoonosis with worldwide distribution. Human infection may occur after ingestion of infective eggs passed in the feces from dogs through direct contact or via environmental contamination (1). Echinococcosis is most prevalent in sheep- and cattle-raising regions like Australia, South America, the Middle East, South Africa, Eastern Europe, and the Mediterranean (2).

Although control programs against human cystic echinococcosis (CE) caused by *E. granulosus* have been established in some countries and effective control strategies are available, the parasite still has a wide geographic distribution, affecting many countries of all continents. Thus, human CE persists in many parts of the world with high incidences, and in some areas, it is a reemerging problem. For example, alarming increases in the number of human cases have been reported from Bulgaria, Kazakhstan, and the People's Republic of China (3). On the other hand, human cystic echinococcosis (hydatid disease) continues to be a substantial cause of morbidity and mortality in many parts of the world (4).

Currently four treatment modalities are in use: (i) surgery, (ii) puncture, aspiration, injection of a protoscolicidal agent, and reaspiration (PAIR), (iii) chemotherapy with albendazole (ABZ) or mebendazole (MBZ), and (iv) watching and waiting for inactive, clinically silent cysts. The evidence from carefully designed clinical studies supporting any of the four treatment modalities is insufficient, and choosing treatment options for patients remains controversial (5, 6).

Cystic echinococcosis (CE) is among the most neglected parasitic diseases. Development of new drugs and other treatment modalities receives very little attention, if any, and is slow. Clinical management procedures have evolved over decades without adequate evaluation of important features, such as efficacy, effectiveness, rate of adverse reactions, relapse rate, and cost (6).

Few chemotherapeutic agents are available for the medical management of hydatid disease caused by the parasite *E. granulosus* (7). Benzimidazole carbamate derivatives, such as mebendazole and albendazole, are currently used for chemotherapeutic treatment of CE. Benzimidazoles have to be applied in high daily doses for extended periods of time, and adverse effects, such as leucopenia, elevation of liver transaminases, and alopecia, are frequently observed (8); therefore, a new effective alternative treatment is extremely important in today's climate, where species are becoming resistant, and there has been a resurgence in the use of natural alternative therapies, instead of synthetic pharmaceuticals that often have severe side effects (9).

Zataria multiflora (Shirazi thyme) is an important aromatic plant belonging to the Lamiaceae family, which is distributed in Iran, Afghanistan, and Pakistan (10, 11). Scientific reports show that *Z. multiflora* has anti-inflammatory (12), antiprotozoal (13), antioxidant (14), antibacterial (15, 16), antifungal (17), and scolicidal (18) effects.

Since *Z. multiflora* has a number of medicinal properties, in this experimental study, the *in vivo* efficacy of aromatic water (AW) of *Z. multiflora* in the prevention and treatment of hydatid

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cysts was evaluated in mice experimentally infected with protoscolices of *E. granulosus*. To the best of our knowledge, this is the first report on the antihydatid activity of *Z. multiflora* AW in an animal model.

MATERIALS AND METHODS

Plant material. Aerial parts of *Z. multiflora* Boiss were collected from wild growing plants at the full flowering stage in the Chahak region of the Neyriz suburb, Fars Province, Islamic Republic of Iran, in May 2012. The plant species was identified and authenticated by A. R. Khosravi, a plant taxonomist, at the Shiraz University Herbarium, Shiraz, Iran. A voucher specimen (24984) has been deposited in the herbarium.

AW extraction. The aerial parts of the plant (100 kg of plant material with 400 liters of water) were hydrodistillated for 3 h, using an industrial apparatus from the Shirin Osare Factory. This is the most ancient and versatile method of distillation in Iran and some other countries for producing edible AW and essential oil (EO). In this method, plant materials are fully submerged in water. The water is heated to produce steam carrying the most volatile chemicals. The steam is then chilled (by a condenser), and the resulting distillate is collected. The essential oil will normally float on top of the hydrosol (AW) and then is separated off the AW. The resulted AW was used for gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis.

Extraction of essential oil from AW. To separate essential oil (EO) from AW, 300 ml of the sample was put in a decanter funnel, and active ingredients were extracted by diethyl ether (20 ml of solvent, 4 times). Finally, the solvent was removed with a stream of nitrogen gas. The resulting essential oil was dried over anhydrous sodium sulfate and kept in a sealed vial at low temperature (4°C) until the GC and GC-MS analyses. The yield of essential oil of the AW was 0.12% (wt/vol).

AW essential oil analysis procedure. GC analysis was performed using an Agilent gas chromatograph series 7890-A with a flame ionization detector (FID) to analyze EO obtained from AW. The analysis was carried out on a fused silica capillary HP-5 column (30 m by 0.32-mm inside diameter [i.d.]; film thickness, 0.25 μm). The injector and detector temperatures were kept at 250 and 280°C, respectively. Nitrogen was used as carrier gas at a flow rate of 1 ml/min. The oven temperature was programmed to increase from 60 to 210°C at a rate of 4°C/min, and then the oven was programmed to reach 240°C at a rate of 20°C/min and finally held isothermally for 8.5 min. The split ratio was 1:50. GC-MS analysis was carried out by use of an Agilent gas chromatograph equipped with a fused silica capillary HP-5 MS column (30 m by 0.25-mm i.d.; film thickness, 0.25 μm) coupled with a 5975-C mass spectrometer. Helium was used as a carrier gas with an ionization voltage of 70 eV. The ion source and interface temperatures were 230 and 280°C, respectively. The mass range was from 45 to 550 atomic mass units (amu). The oven temperature program was the same given above for GC.

Identification of compounds. The constituents of the EOs were identified by calculation of their retention indices under temperature-programmed conditions for *n*-alkanes (C_8 to C_{25}) and the oil on a HP-5 column under the same chromatographic conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectrum library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those reported in the literature (19). For quantification purposes, relative area percentages obtained by FID were used without correction factors.

Collection of protoscolices. *E. granulosus* protoscolices were aseptically removed from liver hydatid cysts obtained from naturally infected sheep slaughtered in a Shiraz abattoir in the southern Iran. The hydatid fluid of cysts was aseptically transferred into glass cylinders and left to set for 30 min. The protoscolices settled at the bottom of the cylinders. The supernatant was then removed, and the collected protoscolices were washed several times with sterile 0.85% NaCl and stored in RPMI 1640 medium overnight at 37°C. The viability of protoscolices was assessed by their muscular movements under light microscopy and 0.1% eosin staining.

Infection of mice. Healthy laboratory male mice,10 weeks old and weighing 22 to 24 g, were infected intraperitoneally by injection of 1,500 protoescolices per animal, dissolved in 0.5 ml of medium RPMI 1640. Infected animals were allocated into prevention and therapy groups. All animals in the study were kept at 24 to 25°C, fed *ad libitum*, and given tap water.

Preventive trials. To prove the preventive effect of *Z. multiflora* AW on hydatid cyst formation, the 40 infected animals were allocated into three experimental treatment groups: group a (10 mice) received albendazole at 150 mg/kg body weight/day for 10 days, group b (15 mice) received *Z. multiflora* AW in drinking water at 20 ml/liter for 8 months, and group c (15 mice) were the untreated controls. All remaining mice (8, 10, and 7 mice from groups a, b and c, respectively) were euthanized after 8 months of infection, and necropsy was carried out immediately thereafter.

Therapeutic trials. To prove the therapeutic effect of *Z. multiflora* AW on the hydatid cyst, after 8 months of infection, the remaining infected mice ($n = 15$) were allocated into three experimental treatment groups containing five animals each: group a received albendazole at 300 mg/kg/day for 20 days, group b received *Z. multiflora* AW in drinking water at 40 ml/liter for 30 days, and group c were the untreated controls. All 15 mice were euthanized at the end of the treatment period, and necropsy was carried out immediately thereafter.

At necropsy, the peritoneal cavity was opened, and the internal organs were observed for hydatid cysts. The hydatid cysts were carefully removed. The sizes of the cysts were determined based on a scaled ruler and Adobe Photoshop CS6. The cyst weight was recorded using a digital scale (Kern & Sohn GmbH, Balingen, Germany). The efficacy of *Z. multiflora* AW was evaluated through the comparison of cyst numbers, sizes, and weights and also ultrastructural morphological changes between the three treatment groups.

Electron microscopy. For scanning electron microscopy (SEM), the cysts were sectioned to blocks of about 1 mm^2. The specimens were immersed in 2.5% glutaraldehyde (10 ml)–4% paraformaldehyde (25 ml) in 0.1 M sodium cacodylate buffer (pH 7.2) (50 ml) for 4 h at room temperature, washed twice in 0.1 M sodium cacodylate buffer (each time for 5 min), and postfixed in 2% osmium tetroxide (OsO_4) for 2 h. The samples were then washed two times in distilled water (each time for 5 min). Subsequently, the specimens were dehydrated by sequential incubations in increasing concentrations of ethanol. Dehydrated specimens were finally immersed in hexamethyldisilazane (HMDS) for 20 min and air dried under a fume hood. They were then sputter-coated with gold and examined in a Cambridge S360 scanning electron microscope (Cambridge Instruments, Cambridge, United Kingdom) operating at 20 kV.

Statistical analysis. The weights and sizes of the cysts of different groups (reported as median and interquartile range [IQR]) were compared by Kruskal-Wallis test (nonparametric one-way analysis of variance [ANOVA]). Analysis was done using the Mann-Whitney U test in the SPSS Statistics 11.5 package. Differences of $P < 0.05$ were considered significant.

Ethics. This study conformed to the guidelines for the care and use of laboratory animals established by the Ethics Committee of Shiraz University. Unnecessary animal suffering was avoided throughout the study.

RESULTS

Chemical compositions. The chemical compositions of EO from aromatic water (AW) of *Zataria multiflora* are shown in Table 1. A total of 25 compounds representing 100% of the total oil were identified. Thymol (66.9%), carvacrol (15.2%), and carvone (7.3%) were found to be the major EO constituents. Other constituents were present in very low concentrations (Table 1). A total of 80 mice were included in this experimental work; however,

TABLE 1 Essential oil chemical components of aromatic water of *Zataria multiflora* identified by retention index and GC-MS[a]

Component	% of total (100%)	RI[b]
1-Octen-3-ol	0.1	974
3-Octanone	0.2	983
3-Octanol	0.1	993
p-Cymene	0.1	1,022
1,8-Cineole	1.6	1,029
Linalool	0.4	1,098
Camphor	0.1	1,143
Isomenthone	0.3	1,162
Borneol	0.3	1,163
Terpinene-4-ol	0.6	1,175
p-Cymen-8-ol	0.2	1,183
α-Terpineol	0.8	1,189
neo-Dihydro carveol	2	1,192
cis-Dihydro carvone	1.2	1,195
trans-Dihydro carvone	0.1	1,203
trans-Carveol	0.2	1,217
cis-Carveol	0.1	1,228
Pulegone	1.2	1,238
Carvone	**7.3**	**1,242**
Thymol	**66.9**	**1,294**
Carvacrol	**15.2**	**1,302**
Piperitenone	0.6	1,339
Thymol acetate	0.2	1,352
Butylated hydroxytoluene	0.1	1,510
Spathulenol	0.1	1,575

[a] Results for the major essential oil constituents have been highlighted in boldface for emphasis.
[b] RI, retention index on an HP-5 column.

because of the long duration of the study, some animals died and were excluded from the experiment. The surviving animals were chosen for statistical analysis.

Preventive trials. Aromatic water of *Z. multiflora* (20 ml/liter in drinking water for 8 months) showed a preventive effect on hydatid cyst formation in laboratory mice. The results presented in Table 2 show that no hydatid cyst development occurred in mice treated with *Z. multiflora* AW. The negative effect of *Z. multiflora* AW on hydatid cyst development was similar to that of albendazole (150 mg/kg body weight/day for 10 days).

Therapeutic trials. *Z. multiflora* AW (40 ml/liter in drinking water for 30 days) showed a therapeutic effect on the hydatid cyst. The results presented in Table 3 show that *Z. multiflora* AW has a negative effect on hydatid cyst development. Significant decreases in cyst weights and also cyst sizes compared to those in the untreated control group were obtained in the *Z. multiflora* AW treatment group ($P < 0.05$). The therapeutic effect of *Z. multiflora* AW on hydatid cysts was almost comparable to that of albendazole, and no statistical differences were observed in cyst weights and cyst sizes between the cysts recovered from mice treated with *Z. multiflora* AW and those from mice treated with albendazole ($P > 0.05$).

TABLE 2 Effect of *Zataria multiform* AW and albendazole on prevention of hydatid cyst formation in laboratory mice[a]

Treament group (*n*)	No. of infected mice	Total no. of cysts	Cyst wt (g)		Cyst size (mm)	
			Median[b]	IQR	Median[b]	IQR
Albendazole (8)	0	0	0 B	0	0 B	0
Z. multiflora AW (10)	0	0	0 B	0	0 B	0
Control (7)	5	13	0.015 A	0.50	4.59 A	12.03

[a] Aromatic water (AW) of *Z. multiform* was administered at 20 ml/liter in drinking water for 8 months, and albendazole was administered at 150 mg/kg body weight/day for 10 days.
[b] Different letters show significant difference within each column.

TABLE 3 Effect of *Zataria multiflora* AW and albendazole on treatment of hydatid cyst formation in laboratory mice[a]

Group (*n*)	No. of infected mice	Total no. of cysts	Cyst wt (g)		Cyst size (mm)	
			Median[b]	IQR	Median[b]	IQR
Albendazole (5)	4	8	0.01 B	0.092	2.81 B	2.23
Z. multiflora AW (5)	5	11	0.036 B	0.108	3.10 B	1.48
Control (5)	5	12	0.58 A	0.216	6.72 A	4.12

[a] Aromatic water (AW) of *Z. multiflora* was administered at 40 ml/liter in drinking water for 30 days, and albendazole was administered at 300 mg/kg body weight/day for 20 days.
[b] Different letters show significant difference within each column.

The ultrastructural appearances of the germinal layer after scanning electron microscopy (SEM) analysis of cysts recovered from untreated mice, and mice treated with *Z. multiflora* AW are shown in Fig. 1. No ultrastructural changes (SEM analysis) were observed in the germinal layer of cysts recovered from mice of the untreated control group. In contrast, the germinal layer of cysts recovered from mice treated with *Z. multiflora* AW showed a markedly distorted morphology and an almost completely damaged germinal layer.

DISCUSSION

The control of helminthosis and generally of all parasitic diseases is usually made with synthetic anthelmintics (20). Few chemical agents apart from the benzimidazoles (BZDs) have been used for chemical treatment of hydatidosis, such as nitazoxanide (NTZ) (21). Benzimidazole compounds are the most therapeutic agents that have been used for chemical treatment of hydatidosis. Oxfendazole (7), mebendazole (22, 23), and albendazole (24–27) have been used for the treatment of hydatid disease, but there are several limitations for the formulation of BZD compounds, including their limited water solubility (28).

At this time, albendazole is routinely used pre- and postoperatively in humans and as a treatment of choice for inoperative cystic echinococcosis (29, 30). However, failures such as poor absorption in the intestinal tract, low levels of hepatic concentration, high cost, severe side effects, such as liver toxicity, and resistance have been reported for albendazole (30–34). There are several reports that show combination of albendazole and other scolicidal agents may enhance the antiparasitic effect of this drug against hydatid cyst.

Benzimidazoles exert only a parasitostatic action *in vivo* and have to be given lifelong (35), therefore, a search for new scolicidal agents is needed, and the introduction of new drugs could help patients suffering from cystic echinococcosis (36). According to circumstances and depending on their efficacy, naturally produced plant anthelmintics offer an alternative that can overcome some of these problems and are both sustainable and environmentally acceptable (20).

We have previously studied several medicinal plant extracts or

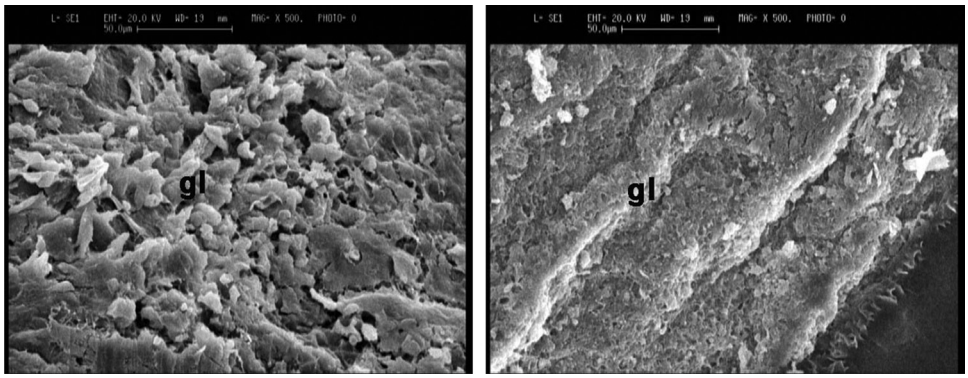

FIG 1 Scanning electron micrographs showing the ultrastructure of the hydatid cysts recovered from untreated infected mice (left) and infected mice treated with the aromatic water (AW) of *Zataria multiflora* (right) 9 months after infection. The left micrograph shows the normal structure of the germinal layer (gl), and the right micrograph shows the distorted morphology and partial lysis of the germinal layer (500×).

essential oils and found that *Z. multiflora* was the most effective agent against protoscolices of hydatid cysts *in vitro* (18, 37–41).

Most of the antimicrobial activity in the essential oils, derived from plants, appears to derive from phenolic compounds. The main constituents of the essential oil of *Z. multiflora* are phenolic compounds, such as carvacrol and thymol (42). In this study, the results of EO analysis of AW showed that *Z. multiflora* EO is a rich source of thymol (66.9%). Similarly, previous studies reported thymol to be the main oil component of *Z. multiflora* (14).

In the present study, it has been confirmed that the AW of *Z. multiflora* has a clear destructive effect on the germinal layer of hydatid cysts in infected mice. Our findings showed that AW of *Z. multiflora* (20 ml/liter in drinking water for 8 months) can prevent hydatid cyst formation in laboratory mice, and this preventive effect was comparable to the preventive effect of albendazole (150 mg/kg/day for 10 days). Furthermore, the results of this study showed that AW of *Z. multiflora* (40 ml/liter in drinking water for 30 days) has a therapeutic effect on hydatid cysts. The therapeutic effect of *Z. multiflora* AW on hydatid cysts was also comparable to that of albendazole. Here, we report our experience in the prevention and treatment of hydatidosis in infected mice following the oral administration of AW of *Z. multiflora*. We observed no hydatid cyst development in the preventive trials and obvious decreases in the weights and sizes of recovered cysts in the therapeutic trials. Our findings are well correlated with scanning electron microscopy analyses, confirming that *in vivo* exposure of hydatid cysts to *Z. multiflora* AW resulted in profound tissue alterations and loss of the cellular structure of the germinal layer. Scanning electron microscopy showed that the germinal layer of hydatid cysts was dramatically damaged following administration of AW of *Z. multiflora* in therapeutic trials.

Spicher et al. observed distorted morphology and partial lysis of the germinal layer-associated tissue in SEM analysis of hydatid cysts recovered from mice treated with 2-methoxyestradiol for 7 days (43). Elissondo et al. observed the killing effect of thymol on the germinal layer of secondary murine hydatid cysts in SEM analysis in an *in vitro* study (44). The results of our study are in agreement with those from the above studies, where we observed clear changes in the germinal layer of hydatid cysts, such as alterations in the germinal layer, massive signs of cellular destruction, detachment of the germinal layer from the laminated layer, the occurrence of a large amount of cellular debris, and the complete absence of the germinal layer.

The germinal layer can contain viable protoscolices (45). Moreover, embryonic or stem cells present on the germinal layer have the potentiality to develop new protoscolices or brood capsules (46). Therefore, for the elimination of hydatid cysts, the germinal layer should be destroyed. Our findings by scanning electron microscopy confirmed the destructive effect of *Z. multiflora* AW on the germinal layer of hydatid cysts. *Z. multiflora* is an edible plant; therefore, it is well adapted to animals and humans. *Z. multiflora* is safe and nonpathogenic to the fetus digestive system when used in pregnant BALB/c mice (47). Furthermore, this herbal plant can remarkably stimulate innate and acquired immunity function in experimental animals, and it may be used as an immunostimulatory agent (48, 49).

Conclusions. The results of this *in vivo* study showed that *Z. multiflora* AW has a destructive effect on the germinal layer of hydatid cyst. These results allowed us to suggest that *Z. multiflora* AW is likely to be a source of useful compounds that could be used as effective antihydatid agents. The results of this study also open the possibility of more investigations of the *in vivo* antihydatid effect of this traditional medicine in human cases of infection.

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